Journal of Fish Diseases 2010, **33**, 559–570 doi:10.1111/j.1365-2761.2010.01152.x

Characterization of susceptibility and carrier status of burbot, *Lota lota* (L.), to IHNV, IPNV, *Flavobacterium psychrophilum*, *Aeromonas salmonicida* and *Renibacterium salmoninarum*

M P Polinski[1], T R Fehringer[1], K A Johnson[2]*, K R Snekvik[3], S E LaPatra[4], B R LaFrentz[5], S C Ireland[6] and K D Cain[1]

1 Department of Fish and Wildlife Resources, College of Natural Resources, University of Idaho, Moscow, ID, USA
2 Idaho Department of Fish and Game, Eagle Fish Health Laboratory, Eagle, ID, USA
3 Washington Animal Disease Diagnostic Laboratory, Department of Veterinary Microbiology & Pathology, Washington State University, Pullman, WA, USA
4 Clear Springs Foods, Inc., Research Division, Buhl, ID, USA
5 United States Department of Agriculture, Agricultural Research Service, Aquatic Animal Health Research Unit, Auburn, AL, USA
6 Kootenai Tribe of Idaho, Bonners Ferry, ID, USA

Abstract

In this study, susceptibility and potential carrier status of burbot, *Lota lota*, were assessed for five important fish pathogens. Burbot demonstrated susceptibility and elevated mortality following challenge with infectious haematopoietic necrosis virus (IHNV) by immersion and to *Aeromonas salmonicida* by intraperitoneal (i.p.) injection. IHNV persisted in fish for at least 28 days, whereas *A. salmonicida* was not re-isolated beyond 17 days post-challenge. In contrast, burbot appeared refractory to *Flavobacterium psychrophilum* following intramuscular (i.m.) injection and to infectious pancreatic necrosis virus (IPNV) by immersion. However, i.p injection of IPNV resulted in re-isolation of virus from fish for the duration of the 28 day challenge. *Renibacterium salmoninarum* appeared to induce an asymptomatic carrier state in burbot following i.p. injection, but overt manifestation of disease was not apparent. Viable bacteria persisted in fish for at least 41 days, and bacterial DNA isolated by diagnostic polymerase chain reaction was detected from burbot kidney tissue 90 days after initial exposure. This study is the first to investigate susceptibility of burbot to selected fish pathogens, and this information will aid in efforts to culture and manage this species.

Keywords: Aeromonas salmonicida, burbot, *Flavobacterium psychrophilum*, IHNV, IPNV, *Renibacterium salmoninarum*.

Correspondence *K D Cain, University of Idaho, Department of Fish & Wildlife Resources, Moscow, ID 83844-1136, USA (e-mail: kcain@uidaho.edu)*
**Retired.*

Introduction

Burbot, *Lota lota* (L.), are the sole true freshwater member of the family Gadidae and can be found in both lacustrian and riverine systems throughout the Holarctic ecozone (McPhail & Paragamian 2000). In recent years, populations have been declining in both North America and Europe. Pulliainen, Korhnonen, Kankaanranta & Maeki (1992) speculated the cause of declining numbers in the Scandinavian coastal regions to be associated with industrial pollution of the aquatic ecosystem. Habitat loss for juvenile rearing in the backwaters of the Mississippi River was cited by Fisher (2000) as a contributing factor to population declines in the region. The decline of the Kootenai River burbot populations of Montana, Idaho and British Columbia to near

Journal of Fish Diseases

extinction appears strongly linked to the alteration in hydrology caused by the construction of the Libby dam (Paragamian 2000; Paragamian, Whitman, Hammond & Andrusak 2000). Because of such population declines, a need to develop conservation aquaculture programmes for burbot has been identified. Specifically, to restore burbot in the Kootenai River, the University of Idaho in cooperation with the Kootenai Tribe of Idaho, Idaho Department of Fish and Game, United States Fish and Wildlife Service, and the British Columbia Ministry of Environment has begun developing hatchery protocols for the artificial culture of burbot as part of a conservation programme for the recovery of this species in the Kootenai River drainage. Research into intensive culture methods has evaluated egg incubation methods (Jensen, Ireland, Siple, Williams & Cain 2008a), captive spawning success (Jensen, Williams, Ireland, Siple, Neufeld & Cain 2008b) and general aquaculture techniques for burbot (Jensen 2006; Jensen, Zuccarelli, Patton, Williams, Ireland & Cain 2008c; Jensen *et al.* 2008a,b). This has resulted in a successful ability for manual collection, fertilization and storage of gametes, egg incubation and larval rearing. As these culture protocols are further characterized, attention must be given to the potential for disease manifestation and transmission in burbot. Such information will be of vital importance for anticipating disease concerns related to intensive culture and hatchery management, as well as for assessing and regulating the consequences of moving burbot beyond their natural migratory distribution or releasing them back into natural environs. Indeed, for conservation aquaculture of burbot to be effective, baseline disease susceptibility status for this species must be understood.

Currently, disease susceptibility of burbot is largely unknown. Manifestations of disease and consequential mortality from an outbreak have not been reported in cultured populations. Burbot have been listed by the United States Department of Agriculture – Animal and Plant Health Inspection Service (USDA-APHIS) as a species susceptible to infection from viral haemorrhagic septicaemia virus genotype IVb found in the Great Lakes Region of the United States and Canada (USDA-APHIS 2007). However, information regarding mortality or the ability of burbot to act as a potential carrier remains unexplored for this or any other viral pathogen. The bacterial pathogen *Yersinia ruckeri* was isolated from burbot in the Mackenzie River of Canada, although manifestations of disease were not apparent (Dwilow, Souter & Knight 1987). In the United States, burbot have tested positive for *Renibacterium salmoninarum* DNA by diagnostic polymerase chain reaction (PCR) from specimens collected in Washington and Montana as part of the U.S. Fish and Wildlife Service Wild Fish Health Survey (USFWS-WFHS 2009). Additionally, *Aeromonas salmonicida*, subsp. *salmonicida* and subsp. *achromogenes*, were also isolated from burbot as part of this survey (USFWS-WFHS 2009). During the captive rearing of wild caught broodstock and progeny (captured in British Columbia) at the University of Idaho, disease sampling conducted at the Washington Animal Disease Diagnostic Laboratory (WADDL) for over 30 mortalities has not detected viral pathogens or bacteria considered to be more than minimal opportunistic bacteria (*Aeromonas caviae* and *Aeromonas hydrophila*). A potentially unique internal fungal infection in adults has been noted, although transmission and associated mortality have yet to be determined (Walsh T & Snekvik KR, unpublished data). Such documentation gives some insight into disease susceptibility of burbot; however, disease manifestations, associated mortality and potential carrier status remain unknown.

Of most concern to regulatory officials involved with Kootenai River burbot reintroduction are five virulent pathogens known to be present within the region. These include infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), *A. salmonicida*, *F. psychrophilum* and *R. salmoninarum*. Given the seriousness and prevalence of these fish pathogens, the lack of information available regarding their ability to infect or persist in burbot and a developing need for conservation aquaculture for this fish species, the objectives of this study were to (1) assess the ability of these pathogens to produce disease and associated mortality in burbot, and (2) determine whether a carrier state can result in burbot for each selected pathogen.

Materials and methods

Fish and rearing conditions

Two fish species, burbot and rainbow trout, *Oncorhynchus mykiss* (Walbaum), were used for each trial to compare the susceptibility of burbot to each pathogen to that of a positive control, rainbow trout.

All fish used in this study were obtained from the University of Idaho's Aquaculture Research Institute (ARI), Moscow, ID, USA, and were maintained in tanks (as described below) supplied with de-chlorinated, single-pass, municipal water. Water temperatures were maintained at 14–15 °C during all rearing and challenge procedures in this study.

Juvenile burbot and rainbow trout. Burbot larvae (< 0.5 g) were maintained in 240 L troughs and were fed *ad libitum* via mechanical belt feeders with commercially prepared 600–800 μm weaning diets (Epac CW; INVE Aquaculture, Inc.). Fish were visually graded by hand every few weeks by size to reduce cannibalism. Once fish reached approximately 1 g, feed size was increased to 800–1200 μm (Epac CW). Portions of these fish were transferred to 19 L circular tanks for all pathogen challenge trials except for those involving *R. salmoninarum*. Juvenile rainbow trout (< 1 g) having no previous disease history were maintained in 200-L circular tanks and were fed a crumbled trout diet (Rangen, Inc.) once daily to apparent satiation. Portions of these fish were also transferred to 19-L tanks for challenge trials. During challenge, burbot and rainbow trout were fed Epac CW 800–1200 μm and a Rangen crumbed trout diet once daily to apparent satiation, respectively.

Subadult burbot and rainbow trout. Juvenile burbot were transferred to 200-L circular tanks for grow out. Fish were maintained by feeding Epac 800–1200 μm weaning diet once daily to apparent satiation for approximately 6 months, transitioned to Epac cod diet (2.0 mm) for 6 months, and then transitioned to Rangen 5/32 moist pellet trout diet and fed 1–2% body weight per day for approximately 1 year. A portion of these fish were transferred to 200-L tanks for *R. salmoninarum* challenge trials. Rainbow trout (approximately 70 g) having no previous disease history were obtained from the ARI on the day of challenge. During challenge, both burbot and rainbow trout were maintained by feeding Rangen 5/32 moist pellet trout diet to apparent satiation once daily.

Pathogen preparation and quantification

A summary of pathogens used to challenge burbot and rainbow trout in this study are included in Table 1. Each pathogen was prepared and quantified for fish challenge as described below.

IHNV. Isolates representing both M and U genotypes of IHNV (Kurath, Garver, Troyer, Emmenegger, Einer-Jensen & Anderson 2003) were propagated on *Epithelioma papulosum cyprini* cells (EPC; Fijan, Sulimanovic, Béarzotti, Musinic, Zwillenberg, Chilmonczyk, Vantherot & de Kinkelin 1983) at 15 °C in minimal essential media (MEM) with Earle's salts supplemented with foetal bovine serum and Glutamax, pH 7.4 (MEM-10; GIBCO® cell culture, Invitrogen Corporation) as described by Anderson, Engelking, Emmenegger & Kurath (2000) with slight modifications. Briefly, cells were infected with each viral isolate at a multiplicity of infection of 0.01. Once complete cytopathic effect (CPE) was observed (4–7 days), culture media was removed, clarified by centrifugation at 5000 ***g*** for 5 min at 15 °C and stored at −80 °C. Upon the day of challenge, viral stocks were thawed at 15 °C in a water bath and diluted in culture water for waterborne exposure. Quantification of virus was accomplished by plaque assay procedures similar to those of LaPatra, Barone, Jones & Zon (2000) where virus was inoculated in replicate with serial $\log_{10}$ dilutions onto EPC cell culture and allowed to absorb for 60 min. Growth media (MEM-10) supplemented with 1% methyl cellulose was added and after 7-days incubation at 15 °C, cells were fixed with formalin, stained with 1% crystal violet solution, and plaques were enumerated.

IPNV. Two North American serotypes of IPNV were obtained to represent the West Buxton A1 and Jasper A9 serotypes (Hill & Way 1995). Virus was propagated in a similar manner to IHNV using Chinook salmon embryo (CHSE-214; Lannan, Winton & Fryer 1984) cell cultures. Virus was quantified at challenge on CHSE-214 cell culture assays after 7 days of incubation at 15 °C by the tissue culture infective dose 50% ($TCID_{50}$) method of Reed & Muench (1938).

Flavobacterium psychrophilum. A virulent isolate of *F. psychrophilum* (259-93) was cultured on tryptone yeast extract salts (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulphate, pH 7.2) agar at 15 °C for 5 days as described by LaFrentz, Lapatra, Call & Cain (2008). Bacterial cells were harvested by centrifugation at 6500 ***g*** for 15 min at 4 °C, and the pellet was re-suspended in phosphate-buffered saline (PBS) to an optical density (OD) of 0.09 and 0.17 at 525 nm

Table 1 Summary of pathogen isolates used for challenge. Challenge method indicated for waterborne immersion (W), intraperitoneal injection (i.p.), or intramuscular injection (i.m.); NA = previous citation not available. Challenge dose given as amount of pathogen administered to individual burbot during challenge; high and low dose are indicated if more than one concentration was used

Pathogen	Isolate	Host species	Location	Year	Citation	Challenge method	Challenge dose (burbot^{-1})
IPNV (serotype A9)	CF-94	Cutthroat trout *Oncorhynchus clarki*	Clark Fork Hatchery, ID, USA	1994	NA	W/i.p.	2.5×10^5 $TCID_{50}$
IPNV (serotype A1)	Buhl-93	Rainbow trout *Oncorhynchus mykiss*	Buhl, ID, USA	1993	(LaPatra *et al.*1993b)	i.p.	2.5×10^5 $TCID_{50}$
IHNV (genotype U)	RB1	Steelhead trout *O. mykiss*	Round Butte Hatchery, OR, USA	2000	(Anderson *et al.* 2000)	W	9.5×10^4 PFU
IHNV (genotype M)	220-90	Rainbow trout *O. mykiss*	Buhl, ID, USA	1990	(LaPatra *et al.*1994)	W	8.8×10^4 PFU
Flavobacterium psychrophilum	259-93	Rainbow trout *O. mykiss*	Southern Idaho, USA	1993	(Sudheesh *et al.*2007)	i.m.	1.0×10^6 CFU (low) 2.5×10^6 CFU (high)
Aeromonas salmonicida salmonicida	HN-00	Rainbow trout *O. mykiss*	Hagerman National Hatchery, ID, USA	2000	NA	i.p.	2.1×10^8 CFU
Renibacterium salmoninarum	CK-90	Steelhead trout *O. mykiss*	Dworshak National Hatchery, ID, USA	1990	(Jones *et al.* 2007)	i.p.	5.1×10^8 cells (low) 1.7×10^9 cells (high)

IPNV, infectious pancreatic necrosis virus; IHNV, infectious haematopoietic necrosis virus.

for a low and high challenge dose, respectively. Bacteria were quantified at challenge using the drop plate method (Chen, Nace & Irwin 2003).

Aeromonas salmonicida. Isolate HN-00 of *A. salmonicida* subsp. *salmonicida* was cultured on tryptic soy agar (TSA; Becton Dickinson) for 48 h at 23 °C. Three colonies were used to inoculate 200 mL tryptic soy broth (TSB) which was incubated statically at 23 °C for 48 h. Cells were harvested by centrifugation at 1000 ***g*** for 15 min at 4 °C and re-suspended in PBS to an OD of 0.98 at 525 nm. Bacteria were quantified at challenge using the drop plate method (Chen *et al.* 2003).

Renibacterium salmoninarum. Isolate CK-90 of *R. salmoninarum* was cultured in 100 mL of KDM-2 (kidney disease medium) broth (Evelyn 1977) for 14 days at 15 °C. Ten microlitres of this culture was used to inoculate slants of KDM-C agar (Daly & Stevenson 1985) and cultured for 17 days at 15 °C. Bacteria were removed from slants by suspension in PBS through gentle titration. Cells were harvested by centrifugation at 1000 ***g*** for 15 min at 4 °C and re-suspended in PBS to an OD of 1.75 and 1.0 at 525 nm for high and low challenge doses, respectively. Bacteria were enumerated using a Petroff-Hausser counting chamber immediately following challenge.

Challenge procedures

IHNV and IPNV immersion. Fish were challenged with IHNV (220-90 and RB1) and IPNV (CF-94) by immersion similar to methods described by LaPatra, Turner, Lauda, Jones & Walker (1993c). Triplicate 25-fish groups of juvenile burbot (mean weight 1.6 g) and rainbow trout (mean weight 0.8 g) were exposed to waterborne IHNV at approximately10^5 plaque-forming units (PFU) per mL or IPNV at approximately 10^5 $TCID_{50}$ mL^{-1} in an amount of water equalling 10 times the total weight (g) of the fish for 60 min in closed systems with aeration. Each group was then placed in a separate 19-L aquarium. An additional triplicate group of each fish species was mock infected by immersion containing MEM-10 without virus to act as a negative control.

IPNV injection. Burbot (mean weight, 0.5 g) and rainbow trout (mean weight, 1.4 g) were challenged with IPNV (CF-94 and Buhl-93) by injection. For

both species, triplicate 20-fish groups were anaesthetized by immersion in 100 μg mL^{-1} tricaine methane sulphonate (MS-222; Argent). Fish were given 25 μL (burbot) or 50 μL (rainbow trout) suspensions of CF-94 and Buhl-93 viral isolates by intraperitoneal (i.p.) injection of 1.0×10^7 $TCID_{50}$ mL^{-1} viral suspension on the left ventral surface just anterior to the anus using a 30-gauge needle. Each group was then placed in a separate 19-L aquarium. An additional triplicate group of each fish species was mock infected by injection of MEM-10 without virus to act as a control.

Flavobacterium psychrophilum. Juvenile burbot (mean weight 4.6 g) and rainbow trout (mean weight 4.9 g) were challenged following standard challenge procedures with slight modifications (LaFrentz, Lapatra, Jones & Cain 2003). Briefly, fish were anaesthetized by immersion in 100 μg mL^{-1} MS-222 and administered 25 μL *F. psychrophilum* 259-93 by intramuscular (i.m.) injection at a low and high challenge dose of 4.0×10^7 and 9.9×10^7 colony-forming units (CFU) per mL, respectively, on the left anterior side of the caudal peduncle using a 30-gauge needle. Fish were administered bacteria in duplicate 20-fish groups. Each group was then placed in a separate 19-L aquarium, and an additional duplicate group of each fish species was similarly mock infected using PBS as a control.

Aeromonas salmonicida. Duplicate 20-fish groups of juvenile burbot (mean weight 3.5 g) and rainbow trout (mean weight 2.1 g) were anaesthetized by immersion in 100 μg mL^{-1} MS-222 and challenged using a standard injection model (Burr, Pugovkin, Wahli, Segner & Frey 2005; Dacanay, Knickle, Solanky, Boyd, Walter, Brown, Johnson & Reith 2006) where fish were administered 50 μL *A. salmonicida* HN-00 isolate suspension by i.p. injection on the left ventral surface just anterior to the anus using a 30-gauge needle. Each group was then placed in separate 19-L aquaria. An additional duplicate group of each fish species was mock infected with PBS to act as a control.

Renibacterium salmoninarum. Challenges involving *R. salmoninarum* were conducted in a similar manner to those of Jones & Moffitt (2004). Subadult burbot (mean weight, 125 g) and rainbow trout (mean weight, 73 g) were anaesthetized with MS-222 as previously described and administered 150 μL *R. salmoninarum* CK-90 isolate suspension by i.p. injection at high and low challenge doses of 1.1×10^{10} and 3.4×10^9 bacteria mL^{-1}, respectively, on the left ventral surface just anterior to the anus using a 22-gauge needle in duplicate 20-fish groups. Care was taken to continually mix bacterial suspensions during the challenge procedure. Each group was then placed in separate 200-L circular tanks. Low dose and high dose treatments were compared to PBS-injected controls for both fish species.

Monitoring and sampling

Mortalities were recorded and removed daily for 60 days following pathogen exposure for *R. salmoninarum* and 28 days for all other pathogen trials. Pathogen re-isolation was attempted on a minimum of 20% daily mortality from each tank. Re-isolation of pathogens was also attempted at the termination of each challenge on 10–15% (original population size) of surviving fish if available. Procedures for viral and bacterial isolation are as described below.

Virus re-isolation. Whole fish were homogenized in MEM-10 supplemented with penicillin–streptomycin–neomycin antibiotic mixture (0.05 mg, 0.05 mg, 0.1 mg mL^{-1}, respectively; GIBCO®) in a volume equaling 10 times the weight (g) of fish. Homogenates were centrifuged at 3000 ***g*** for 10 min at 15 °C to pellet debris. Remaining supernatants were forced through 0.45-μm syringe filters (Fisher Scientific®). Filtrate was collected and used directly for quantification assays or stored at −80 °C for later use in reverse transcriptase PCR (RT-PCR). Viral quantification was performed as previously described. If CPE was observed in the cell culture assay, RT-PCR was performed on the original sample filtrate to confirm IHNV or IPNV by standard methods (USFWS and AFS-FHS 2005).

Bacteria re-isolation. Isolation of bacteria was attempted from sampled fish using standard methods (USFWS and AFS-FHS 2005). Briefly, kidney, liver and spleen tissues were inoculated onto TSA or KDM-C agar for the isolation of *A. salmonicida* and *R. salmoninarum*, respectively. Spleen tissue was inoculated onto TYES agar for the isolation of *F. psychrophilum*. Cultures were

incubated at 15 °C for up to 14 days for *A. salmonicida* and *F. psychrophilum* or as long as 60 days for *R. salmoninarum*. Resulting colonies were identified using morphological characteristics and Gram staining.

Statistical analysis

Statistical analysis of data was accomplished using GraphPad Prism 2.01 software. Survival curves were generated to analyse mortality rate by the Kaplan–Meier method (Kaplan & Meier 1958). Curves were compared using the logrank test (Petro & Petro 1972), and differences were considered significant at P-value < 0.05.

Results

IHNV

Waterborne challenge of burbot with the 220-90 and RB1 strains of IHNV resulted in significantly different survival curves post-infection (Fig. 1a). Mortality in challenged burbot was first observed 5 days post-challenge and continued through to the termination of the trial. Burbot showed a less severe and more protracted infection with both IHNV isolates than rainbow trout. Challenge of rainbow trout with 220-90 and RB1 resulted in mean mortality of 99% and 39%, respectively, where all mortality occurred between 4 and 13 days post-challenge. In challenged burbot, viral titres ranged from 10^4 to $> 10^6$ PFU per fish, and IHNV was confirmed by RT-PCR. However, no external or internal clinical signs of disease could be distinguished relative to mock-infected fish. Surviving burbot following challenge with 220-90 yielded viral titres in the range of 10^2–10^4 PFU per fish, but virus was not detected in cell culture or by PCR from surviving burbot challenged with the RB1 isolate. Viral titres in rainbow trout were $> 10^6$ PFU per fish in all samples for 220-90 and ranged from 10^5 to $> 10^6$ PFU per fish in samples for RB1. Rainbow trout surviving challenge with RB1 had viral titres between 10^3 to $> 10^6$ PFU per fish at 28 days. Only a single rainbow trout survived challenge with 220-90, from which virus was not detected.

IPNV

Challenge with the CF-94 isolate of IPNV by immersion did not appear to induce disease in burbot and was not re-isolated from challenged mortalities or survivors (Table 2). There were no significant differences between the survival curves of the infected and control fish. Mortality in rainbow trout was not observed (Fig. 1b); however, viral titres in surviving rainbow trout following challenge ranged from 10^2 to 10^3 $TCID_{50}$ per fish, indicating that a latent viral infection was induced in this species.

Injection challenge with CF-94 and Buhl-93 isolates of IPNV did not induce clinical signs of disease in burbot or appear to significantly effect mortality (Fig. 1c). However, unpredictable survival inherent in the culture of this early life stage for burbot (see Discussion) resulted in 100% mortality in mock infected controls making pathogen induced mortality difficult to access. Nevertheless, viable virus was cultured from mortalities and survivors following challenge (Table 2), indicating this ability of the virus to persist in burbot for at least 28 days. It remains unclear as to whether this persistence was due to injected virus remaining viable or resulted from *in vivo* viral propagation as quantification of virus beyond original challenge levels was not attempted.

Flavobacterium psychrophilum

Challenge of rainbow trout by i.m. injection of *F. psychrophilum* induced severe infections with near 100% mortality (Fig. 1d). Lesions developed typical of experimental coldwater disease (CWD), and *F. psychrophilum* was re-isolated from the spleen of all the rainbow trout mortalities that were examined. In contrast, burbot exhibited no overt disease manifestations, and *F. psychrophilum* was not cultured from challenged mortalities or survivors (Table 2). Survival curves of burbot challenged with the high and low doses were not significantly different from that of the mock-infected controls (Fig. 1d).

Aeromonas salmonicida

Burbot demonstrated a susceptibility to *A. salmonicida* subsp. *salmonicida*. There was significant difference in the survival curves between challenged and mock-infected groups. Infected burbot developed lesions around the injection site and became lethargic with a poor feeding response. Upon necropsy, petechial haemorrhaging was observed on internal organs and peritoneal walls.

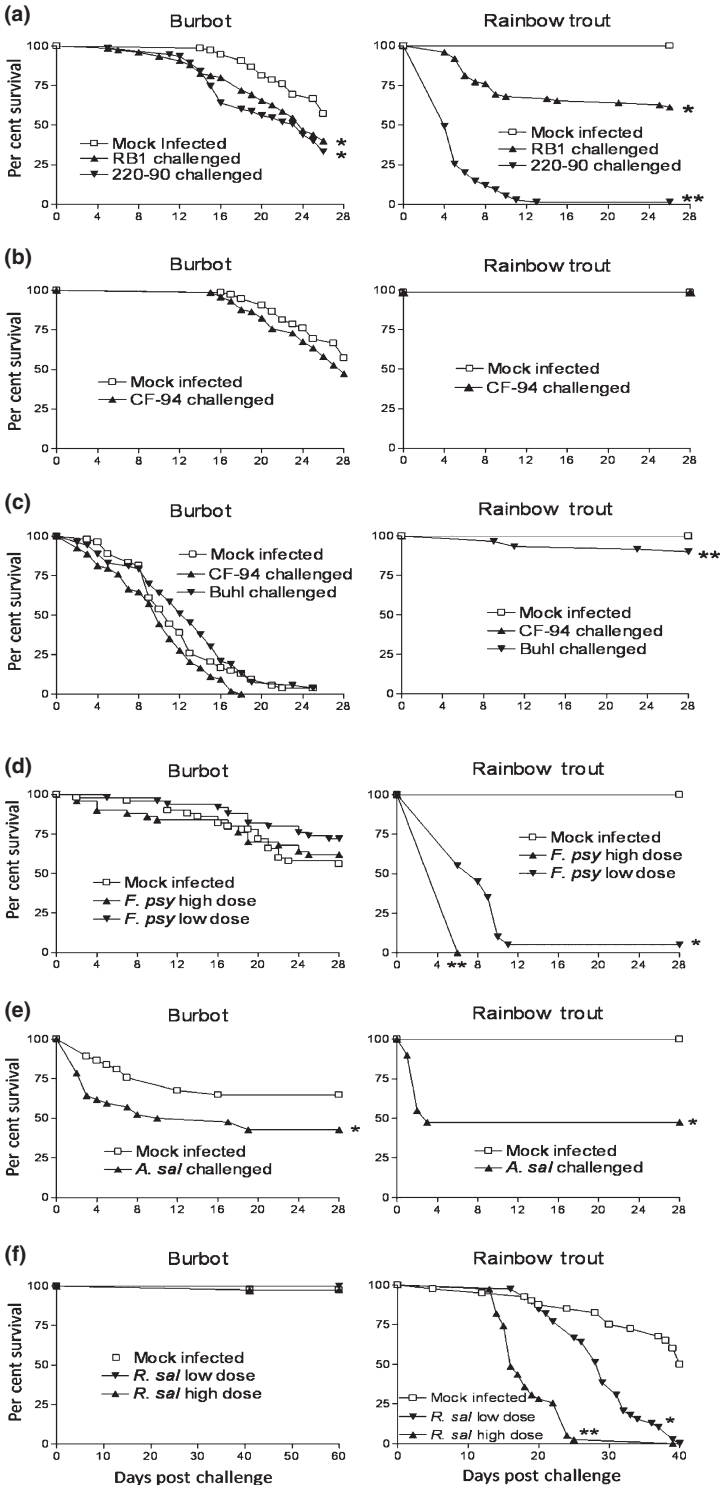

Figure 1 Curves comparing survival rates of burbot and rainbow trout following challenge with infectious haematopoietic necrosis virus by immersion (a), infectious pancreatic necrosis virus (IPNV) by immersion (b), IPNV by i.p. injection (c), *Flavobacterium psychrophilum* by i.m. injection (d), *Aeromonas salmonicida salmonicida* by i.p. injection (e) and *Renibacterium salmoninarum* by i.p. injection (f). Asterisk (*) indicates significant increased mortality rate relative to mock-infected controls within each trial, asterisks (**) indicate significant increased mortality rate relative to other treatments as well as controls within each trial.

Table 2 Pathogen re-isolation from challenged burbot and rainbow trout. Re-isolation of appropriate pathogen (+), pathogen was not re-isolated (–), or samples were unavailable (NA) to attempt re-isolation. Challenge methods are indicated for waterborne immersion (W), intraperitoneal injection (i.p.), or intramuscular injection (i.m.)

				Pathogen re-isolation	
Pathogen	Isolate	Challenge method	Species challenged	Mortalities	Survivors
IPNV (serotype A9)	CF-94	W	Burbot	–	–
			Rainbow trout	NA	+
IPNV (serotype A9)	CF-94	i.p.	Burbot	+	+
			Rainbow trout	NA	+
IPNV (serotype A1)	Buhl-93	i.p.	Burbot	+	+
			Rainbow trout	+	+
IHNV (genotype U)	RB1	W	Burbot	+	–
			Rainbow trout	+	+
IHNV (genotype M)	220-90	W	Burbot	+	+
			Rainbow trout	+	+
Flavobacterium psychrophilum	259-93	i.m.	Burbot	–	–
			Rainbow trout	+	+
Aeromonas salmonicida salmonicida	HN-00	i.p.	Burbot	+	–
			Rainbow trout	+	+
Renibacterium salmoninarum	CK-90	i.p.	Burbot	+	+
			Rainbow trout	+	+

IPNV, infectious pancreatic necrosis virus; IHNV, infectious haematopoietic necrosis virus.

Gram-negative, non-motile, bacterial colonies having brown diffusible pigment characteristic of *A. salmonicida* subsp. *salmonicida* were isolated from the kidney, liver and spleen of challenged burbot mortalities (Table 2). Burbot appeared less sensitive to disease caused by *A. salmonicida* than rainbow trout, as higher and more acute mortality was observed in rainbow trout compared to burbot (Fig. 1e). Additionally, the bacterium was cultured from tissues of surviving rainbow trout following challenge, whereas *A. salmonicida* was not detected in surviving burbot (Table 2).

Renibacterium salmoninarum

Challenge of burbot with *R. salmoninarum* did not induce disease or mortality, although a latent carrier state developed. Mortality during the 60-day trial was minimal, and the survival curves were not significantly different between burbot challenged with both doses of *R. salmoninarum* and the mock-infected control group (Fig, 1f). The one mortality that occurred in burbot (high dose treatment) at 41 days post-challenge yielded growth of *R. salmoninarum* in culture from kidney, liver and spleen, demonstrating viable bacteria can persist in these tissues of burbot. *Renibacterium salmoninarum* was not cultured from tissue of any other burbot sampled; however, diagnostic nested PCR of kidney tissues from surviving fish revealed the presence of *R. salmoninarum* DNA in these tissues up until the termination of the challenged stocks (90 days post-infection). Conversely, rainbow trout experienced typical manifestations of bacterial kidney disease (BKD) caused by *R. salmoninarum* with 100% mortality at both doses. Survival curves of rainbow trout challenged with both doses of *R. salmoninarum* were significantly different from the mock-infected control group (Fig. 1f). *Renibacterium salmoninarum* was cultured from liver, kidney and spleen from 46% (18/39) of rainbow trout challenge mortalities examined but not from mock-infected fish. Causes of mortality in mock-infected rainbow trout during this trial were undetermined.

Discussion

The development of aquaculture methods for a new species is challenging and brings with it difficulties inherent for dealing with an unknown. Attempting to optimize performance of new species in captivity can be problematic and protocols for the intensive culture of burbot have only just begun to be developed (Jensen *et al.* 2008a). General observations at the ARI burbot aquaculture rearing facility have noted high losses during early larval rearing of burbot with chronic mortality often persisting into juvenile development (Jensen NR & Cain KD, unpublished data). This is not uncommon for highly fecund species possessing a larval stage that must be weaned from live to artificial diets. Techniques are improving, but early life stage

rearing of burbot is extremely delicate. Ideally, studies into disease susceptibility would be conducted once fish mortality had stabilized; however, the numbers of individuals required to conduct adequate replicated studies currently surpasses available stock populations reaching such stability. Furthermore, the potential mortality and manifestations of disease exclusively associated with young fish, such as seen with IPNV in salmonids (Reno 1999; LaPatra *et al.* 2000), would likely be missed. Therefore, in the majority of these trials, stable population survival was sacrificed (as observed by mortality in mock-infected controls) to ensure adequate fish numbers at a size where disease manifestations would most likely be apparent. This was an unfortunate sacrifice, as a clear picture of pathogen-induced mortality was thus confounded. However, the comparisons of survival rate between mock-infected and challenged fish, as well as the investigation into pathogen re-isolation, provide a solid foundation to determine gross levels of susceptibility; the primary objective of this research.

Results in this study suggest that burbot are susceptible to the Novirhabdovirus IHNV because of the increased mortality observed after waterborne challenge with both M and U genotypes of IHNV and the presence of viable virus in fish for up to 28 days post-exposure. Previous studies have shown a variation in virulence for these genotypes depending on host species. LaPatra, Fryer & Rohovec (1993a) found experimentally infecting 0.4 g rainbow trout with U or M isolates resulted in 30% and 100% cumulative percent mortality (CPM), respectively. Similar results were observed with rainbow trout in this study (39% CPM U, 99% CPM M). Challenge of burbot with these genotypes resulted in similar mortality from both viral genotypes, and there was a lower increased mortality than observed in rainbow trout. This suggests that burbot are less susceptible to either of these isolates than rainbow trout. It is interesting to note when considering the ability of burbot to act as a potential vector of IHNV that, in this study, visually asymptomatic burbot 28 days following challenge with the M genotype isolate were infected with high levels of viable virus ($> 10^6$ PFU per fish) in some cases. No virus was re-isolated from burbot survivors following challenge with the U genotype isolate. It has been hypothesized that *in vivo* virulence of IHNV is linked to the ability for rapid replication by the virus, thus allowing a kinetic advantage over the host innate immune response (Purcell, Garver, Conway, Elliott & Kurath 2009; Penaranda, Purcell & Kurath 2009). Although no attempts were made to evaluate an immune response in this study, it is evident that both IHNV isolates have the potential to quickly overcome the burbot innate immune response as seen by high viral titres in challenged mortalities within 5 days post-infection. However, there were differences between the genotypes in regard to pathogen persistence, where burbot appeared to be able to clear (or at least drastically reduce) infection from the U genotype while the M genotype continued to persist at high levels. This may suggest that the M genotype of IHNV has a slightly higher virulence than the U genotype in burbot. Further investigation is necessary to determine the ability of burbot to transmit this virus; however, given these current findings, burbot should be considered a possible vector for the U and M genotypes of IHNV. Currently, the distribution of the U genotype of IHNV overlaps with endemic burbot populations (Garver, Troyer & Kurath 2003) in the United States and Canada, whereas the distribution of the M genotype is unconnected. If M genotype contact were to occur, the ability for burbot to act as a vector for IHNV could become enhanced.

The susceptibility of burbot to IPNV remains somewhat ambiguous. Waterborne exposure of burbot to IPNV did not increase mortality and a similar result occurred with rainbow trout. Because mortality and disease manifestations of IPNV in salmonids is often only observed between 1 and 4 months of age (LaPatra *et al.* 2000), it is quite possible that the 6-month-old fish challenged in this study were at an age and weight (Okamoto & Sano 1992) that resulted in limited disease manifestations. Nevertheless, latent infection developed in rainbow trout but not in burbot, suggesting that waterborne exposure alone is insufficient to induce viral infection in burbot. In a second challenge trial with IPNV, fish were administered virus by i.p. injection to bypass the external fish barrier. Additionally, this trial used smaller burbot (mean weight, 0.5 g) and an additional isolate (Buhl-93) representing the A1 serotype with known virulence in rainbow trout (LaPatra, Lauda, Woolley & Armstrong 1993b) in an attempt to elicit mortality. However, the results were uninformative concerning burbot because of the high background mortality in the mock-infected controls. Nevertheless, viable IPNV was re-isolated from mortalities and survivors following the challenge indicating

that IPNV can persist *in vivo* for at least 28 days in this species. Because IPNV is known to be extremely environmentally stable (Toranzo & Hetrick 1982), it is unclear as to whether virus re-isolated from challenged fish was because of persistence of the original injected virions or virus replication in the host. Endpoint viral titres for this study were not determined beyond 10^5 $TCID_{50}$ per fish, and as fish were injected with a similar dose, it remains unclear as to whether viral replication occurred. Further investigation will be needed to determine the full extent of susceptibility of burbot to IPNV. Although waterborne exposure, which is presumably the more natural mode of infection, did not result in infection, it may be advisable to consider burbot a possible vector for IPNV given IPNV demonstrated ability to persist *in vivo* in burbot for a considerable period following injection.

Burbot appeared refractory to CWD caused by *F. psychrophilum* following i.m. injection. It is possible that an alternative exposure method, such immersion, could induce disease. However, such methods have proven less effective than injection to induce disease in salmonids (Garcia, Pozet & Michel 2000). Therefore, the failure of *F. psychrophilum* to induce disease manifestations at high injection doses in this trial suggests an overall refractive nature of burbot to this bacterial pathogen and that burbot are unlikely to be a susceptible host.

Burbot were demonstrated to be susceptible to *A. salmonicida* subsp. *salmonicida* by i.p. injection. Lesions developed, mortality rates increased and bacteria were re-isolated from internal organs. Disease manifested to a lesser degree in burbot than observed in rainbow trout. In this trial, rainbow trout surviving initial exposure became asymptomatic carriers, and previous observations have noted such covert infections to be a common occurrence in salmonids (Hiney, Smith & Bernoth 1997). In contrast, burbot that survived the challenge cleared the bacteria to below detectable levels. Further investigation into the ability of burbot to transmit this pathogen and the duration of any transmission is warranted because two *A. salmonicida* subspecies have been isolated from wild burbot stocks (USFWS-WFHS 2009).

Injection of *R. salmoninarum* into burbot induced an asymptomatic carrier state. Manifestations of disease were not apparent even at the high dose used for challenge (1.7×10^9 bacteria per fish); however, viable bacteria were cultured from the single high-dose mortality at 41 days post-exposure, and bacterial DNA was detected by PCR in both high- and low-dose challenge groups at 90 days post-challenge. Previous research has shown that *R. salmoninarum* can survive macrophage phagocytosis and even escape the phagosome to the cellular cytoplasm (reviewed by Wiens & Kaattari 1999). Such intracellular invasion may help to explain the lack of immune clearance of this bacterium in burbot and the persistence of bacterial DNA. Additionally, Hirvela-Koshi, Pohjanvirta, Koski & Sukura (2006) noted atypical growth and morphological characteristics during subclinical infections, suggesting that when conditions are suboptimal, this bacterium may enter a dormant state. Atypical morphology was not observed in the only case where *R. salmoninarum* was cultured from burbot in this study; however, bacterial persistence in some dormant state at relatively low levels could explain the inability to culture this bacteria from other samples from burbot that were positive by PCR.

In this study, we present baseline susceptibility of burbot to five important and prevalent fish pathogens so as to provide a tool for fisheries and fish health managers to address concerns of pathogen transfer for this species. Burbot demonstrated a susceptibility to IHNV as well as *A. salmonicida*. Burbot appeared refractory to CWD caused by *F. psychrophilum* and IPN caused by IPNV. Burbot did not appear susceptible to BKD caused by *R. salmoninarum*, but a latent carrier state appeared to develop. This study lays a foundation for further investigation into the ability of burbot to serve as potential vectors for these pathogens as well as insight into potential disease concerns related to this species both in future aquaculture or in natural environments.

Acknowledgements

Funding for this work was provided by the Kootenai Tribe of Idaho through contracts with the Bonneville Power Administration (Project 198806400/Contract 30729 and 37267). We thank staff at the Aquaculture Research Institute for providing fish and assisting in care during this study; particularly Nathan Jensen, Scott Barnes and Chris Thorton. We also thank Christine Moffitt, David Burbank and Amy Long for aiding in challenge procedures. We gratefully acknowledge staff at Clear Springs Foods, specifically Bill Shewmaker and Merle Higgins as well as Bill Batts and Gael Kurath at the US

Geological Survey Western Fish Disease Laboratory, and Laura Kessel and Kathy Clemens at the Idaho Department of Fish and Game Idaho Fish Health Center for providing viral isolates and giving constructive advice for the development of experimental methods used in this study.

References

Anderson E.D., Engelking H.M., Emmenegger E.J. & Kurath G. (2000) Molecular epidemiology reveals emergence of a virulent infectious haematopoietic necrosis (IHN) virus strain in wild salmon and its transmission to hatchery fish. *Journal of Aquatic Animal Health* **12,** 85–99.

Burr S.E., Pugovkin D., Wahli T., Segner H. & Frey J. (2005) Attenuated virulence of an *Aeromonas salmonicida* subsp. *salmonicida* type III secretion mutant in a rainbow trout model. *Microbiology* **151,** 2111–2118.

Chen C.-Y., Nace G.W. & Irwin P.L. (2003) A 6x6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*. *Journal of Microbiological Methods* **55,** 475–479.

Dacanay A., Knickle L., Solanky K.S., Boyd J.M., Walter J.A., Brown L.L., Johnson S.C. & Reith M. (2006) Contribution of the type III secretion system (TTSS) to virulence of *Aeromonas salmonicida* subsp. *salmonicida*. *Microbiology* **152,** 1847–1856.

Daly J.G. & Stevenson R.M.W. (1985) Charcoal agar, a new growth medium for the fish disease bacterium *Renibacterium salmoninarum*. *Applied and Environmental Microbiology* **50,** 868–871.

Dwilow A.G., Souter B.W. & Knight K. (1987) Isolation of *Yersinia ruckeri* from burbot, *Lota lota* (L.), from the Mackenzie River, Canada. *Journal of Fish Diseases* **10,** 315–317.

Evelyn T.P.T. (1977) An improved growth medium for the kidney disease bacterium and some notes on using the medium. *Bulletin de L'Office International des Epizooties* **87,** 511–513.

Fijan N., Sulimanovic D., Béarzotti M., Musinic D., Zwillenberg L.D., Chilmonczyk S., Vantherot J.F. & de Kinkelin P. (1983) Some properties of *epithelioma papulosum cyprini* (EPC) cell line from carp *Cyprinus carpio*. *Annals of Virology* **134,** 207–220.

Fisher S.J. (2000) Early life history observations of burbot utilizing two Missouri River backwaters. In: *Burbot Biology, Ecology, and Management* (ed. by V.L. Paragamian & D.W. Willis), pp. 96–102. Fisheries Management Section of the American Fisheries Society, Spokane.

Garcia C., Pozet F. & Michel C. (2000) Standardization of experimental infection with *Flavobacterium psychrophilum,* the agent of rainbow trout *Oncorhynchus mykiss* fry syndrome. *Diseases of Aquatic Organisms* **42,** 191–197.

Garver K.A., Troyer R.M. & Kurath G. (2003) Two distinct phylogenetic clades of infectious hematopoietic necrosis virus overlap within the Columbia River basin. *Diseases of Aquatic Organisms* **55,** 187–203.

Hill B.J. & Way K. (1995) Serological classification of infectious pancreatic necrosis (IPN) virus and other aquatic birnaviruses. *Annual Review of Fish Diseases* **5,** 55–77.

Hiney M., Smith P. & Bernoth E.-M. (1997) Covert *Aeromonas salmonicida* infections. In: *Furunculosis: Multidisciplinary Fish Disease Research* (ed. by E.-M. Bernoth, A.E. Ellis, P.J. Midtlyng, G. Olivier & P. Smith), pp. 54–97. Academic Press, London.

Hirvela-Koshi V., Pohjanvirta T., Koski P. & Sukura A. (2006) Atypical growth of *Renibacterium salmoninarum* in subclinical infections. *Journal of Fish Diseases* **29,** 21–29.

Jensen N.R. (2006) Development of aquaculture techniques for burbot: implications for conservation aquaculture and restoration of burbot in Idaho's Kootenai River. Masters Thesis, Department of Fish and Wildlife Resources, University of Idaho, Moscow.

Jensen N.R., Ireland S.C., Siple J.T., Williams S.R. & Cain K.D. (2008a) Evaluation of egg incubation methods and larval feeding regimes for North American burbot. *North American Journal of Aquaculture* **70,** 162–170.

Jensen N.R., Williams S.R., Ireland S.C., Siple J.T., Neufeld M.D. & Cain K.D. (2008b) Preliminary captive burbot spawning observations. In: *Burbot: Ecology, Management, and Culture* (ed. by V.L. Paragamian & D.H. Bennett), pp. 155–166. Fisheries Management Section of the American Fisheries Society, Spokane.

Jensen N.R., Zuccarelli M.D., Patton S.J., Williams S.R., Ireland S.C. & Cain K.D. (2008c) Cryopreservation and methanol effects on burbot sperm motility and egg fertilization. *North American Journal of Aquaculture* **70,** 38–42.

Jones D.T. & Moffitt C.M. (2004) Swimming endurance of bull trout, lake trout, Arctic char, and rainbow trout following challenge with *Renibacterium salmoninarum*. *Journal of Aquatic Animal Health* **16,** 10–22.

Jones D.T., Moffitt C.M. & Peters K.K. (2007) Temperature-mediated differences in bacterial kidney disease expression and survival in *Renibacterium salmoninarum*-challenged bull trout and other salmonids. *North American Journal of Fisheries Management* **27,** 695–706.

Kaplan E.L. & Meier P. (1958) Nonparametric estimation from incomplete observations. *Journal of the American Statistical Association* **53,** 457–481.

Kurath G., Garver K.A., Troyer R.M., Emmenegger E.J., Einer-Jensen K. & Anderson E.D. (2003) Phylogeography of infectious haematopoietic necrosis virus in North America. *Journal of General Virology* **84,** 803–814.

LaFrentz B.R., Lapatra S.E., Jones G.R. & Cain K.D. (2003) Passive immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease and rainbow trout fry syndrome. *Journal of Fish Diseases* **26,** 377–384.

LaFrentz B.R., Lapatra S.E., Call D.R. & Cain K.D. (2008) Isolation of rifampicin resistant *Flavobacterium psychrophilum* strains and their potential as live attenuated vaccine candidates. *Vaccine* **26,** 5582–5589.

Lannan C.N., Winton J.R. & Fryer J.L. (1984) Fish cell lines: establishment and characterization of nine cell lines from salmonids. *In Vitro* **20,** 671–679.

LaPatra S.E., Fryer J.L. & Rohovec J.S. (1993a) Virulence comparison of different electrophero-types of infectious

hematopoietic necrosis virus. *Diseases of Aquatic Organisms* **16,** 115–120.

LaPatra S.E., Lauda K.A., Woolley M.J. & Armstrong R. (1993b) Detection of a naturally occurring coinfection of IHNV and IPNV in rainbow trout. *American Fisheries Society/ Fish Health Section Newsletter* **21,** 9–10.

LaPatra S.E., Turner T., Lauda K.A., Jones G.R. & Walker S. (1993c) Characterization of the humoral response of rainbow trout to infectious hematopoietic necrosis virus. *Journal of Aquatic Animal Health* **5,** 165–171.

LaPatra S.E., Lauda K.A. & Jones G.R. (1994) Antigenic variants of infectious hematopoietic necrosis virus and implications for vaccine development. *Diseases of Aquatic Organisms* **20,** 119–126.

LaPatra S.E., Barone L., Jones G.R. & Zon L.I. (2000) Effects of infectious haematopoietic necrosis virus and infectious pancreatic necrosis virus infection on haematopoietic precursors of the zebrafish. *Blood Cells, Molecules, and Diseases* **26,** 445–452.

McPhail J.D. & Paragamian V.L. (2000) Burbot biology and life history. In: *Burbot Biology, Ecology, and Management* (ed. by V.L. Paragamian & D.W. Willis), pp. 11–22. Fisheries Management Section of the American Fisheries Society, Spokane.

Okamoto N. & Sano T. (1992) Studies on the generation of infectious pancreatic necrosis. In: *Salmonid Diseases* (ed. by T. Kimura), pp. 41–47. University Press, Japan.

Paragamian V.L. (2000) The effects of variable flows on burbot spawning migrations in the Kootenai River, Idaho, USA, and Kootenay Lake, British Columbia, Canada, after construction of the Libby Dam. In: *Burbot Biology, Ecology, and Management* (ed. by V.L. Paragamian & D.W. Willis), pp. 111–126. Fisheries Management Section of the American Fisheries Society, Spokane.

Paragamian V.L., Whitman V., Hammond J. & Andrusak H. (2000) Collapse of burbot fisheries in the Kootenai River, Idaho, USA, and Kootenay Lake, British Columbia Canada. In: *Burbot Biology, Ecology, and Management* (ed. by V.L. Paragamian & D.W. Willis), pp. 155–164. Fisheries Management Section of the American Fisheries Society, Spokane.

Penaranda M.D., Purcell M.K. & Kurath G. (2009) Differential virulence mechanisms of infectious hematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*) include host entry and virus replication kinetics. *Journal of General Virology* **90,** 2172–2182.

Petro R. & Petro J. (1972) Asymptotically efficient rank invariant test procedures. *Journal of the Royal Statistical Society. Series A* **135,** 185–207.

Pulliainen E., Korhnonen K., Kankaanranta L. & Maeki K. (1992) Non-spawning burbot on the northern coast of the Bothnian Bay. *Ambio* **21,** 170–175.

Purcell M.K., Garver K.A., Conway C., Elliott D.G. & Kurath G. (2009) Infectious haematopoietic necrosis virus genogroup-specific virulence mechanisms in sockeye salmon, *Oncorhynchus nerka* (Walbaum), from Redfish Lake, Idaho. *Journal of Fish Diseases* **32,** 619–631.

Reed L. & Muench H. (1938) A simple method of estimating fifty percent endpoints. *American Journal of Hygiene* **27,** 493–497.

Reno P.W. (1999) Infectious pancreatic necrosis and associated aquatic birnaviruses. In: *Fish Diseases and Disorders* Vol. 3 (ed. by P.T.K. Woo & D.W. Bruno), pp. 1–56. CABI Publishing, New York.

Sudheesh P.S., LaFrentz B.R., Call D.R., Siems W.F., LaPatra S.E., Wiens G.D. & Cain K.D. (2007) Identification of potential vaccine target antigens by immunoproteomic analysis of a virulent and a non-virulent strain of the fish pathogen *Flavobacterium psychrophilum*. *Diseases of Aquatic Organisms* **74,** 37–47.

Toranzo A.E. & Hetrick F.M. (1982) Comparative stability of two salmonid viruses and poliovirus in fresh, estuarine and marine waters. *Journal of Fish Diseases* **5,** 223–231.

USDA-APHIS (United States Department of Agriculture-Animal and Plant Health Inspection Service) (2007) *USDA Viral Hemorrhagic Septicemia-Susceptible Species List*. Annapolis, MD.

USFWS and AFS-FHS (U. S. Fish and Wildlife Service and American Fisheries Society-Fish Health Section) (2005) Standard procedures for aquatic animal health inspections. *FHS Blue Book: Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*. AFS-FHS, Bethesda.

USFWS-WFHS (United States Fish and Wildlife Service-Wild Fish Health Survey) (2009) National Wild Fish Health Survey Database. http://www.esg.montana.edu/nfhdb/. Last accessed August 2009.

Wiens G.D. & Kaattari S.L. (1999) Bacterial kidney disease (*Renibacterium salmoninarum*). In: *Fish Diseases and Disorders*, Vol. 3 (ed. by P.T.K. Woo & D.W. Bruno), pp. 269–301. CABI Publishing, New York.

Received: 3 November 2009
Revision received: 11 January 2010
Accepted: 18 January 2010

